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RESEARCH ARTICLE

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Effects of oxidized glutathione, cysteine and taurine supplementations on motility charateristics of different goat spermatozoa types

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ABSTRACT

This study aimed to determine the influences of antioxidants additives on motility characteristics of different goat spermatozoa types including frozen/thawed, fresh, and chilled samples. Ejaculates from five Shami bucks were collected during breeding and non-breeding seasons. Spermatozoa samples from the three types were incubated in media containing 2 and 4 mM oxidized glutathione (GSSG), 5 and 10 mM L-cysteine,10 and 25 mM taurine, and no additives (control). Motility characteristics were analyzed by a computer-aided sperm analyzer (CASA). Except for taurine, the addition of antioxidants resulted in a significant ($p < 0.05$) increase in the percentage of motile sperm (MOT %) after spermatozoa thawing. When fresh sperm samples were collected during the non-breeding season and treated with both GSSG and L-cysteine, the values of the velocity parameters VAP, VSL, and VCL increased significantly ($p < 0.05$). No significant effects were noted for the velocity parameters when 10 and 25 mM of taurine were added to the chilled spermatozoa, while GSSG and L-cysteine had principally affected MOT % of this spermatozoa type. The rapid spermatozoa subpopulation was the most influenced category by the three antioxidants compared to the slow and medium grades, especially in the case of fresh and frozen/thawed types. In conclusion, the effects of different antioxidants on goat spermatozoa motility largely depend on the used concentration and also on the type of spermatozoa pattern.

Keywords

Reactive oxygen species, Antioxidants, Goat, Spermatozoa, Motility.

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Abbreviations

CASA: computer-aided sperm analyzer
CAT: catalase
GSH: glutahione
GSSG: oxidized glutathione
GSH-Px: glutathione peroxidase
GSH: reduced glutathione
GLM: general linear model procedure
MDA: malondialdehyde

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Introduction

It is well known that the sperm plasma membrane is rich in polyunsaturated fatty acids which are susceptible to oxidative stress damage resulting from reactive oxygen species (ROS) during aerobic incubation [1]. To block the effects of oxidative stress, a wide array of antioxidants have been used. Antioxidants could scavenge ROS directly and also they may prevent the propagation of lipid peroxidation in sperm membranes. It must be noted that both spermatozoa and seminal plasma possess antioxidant systems such as taurine, cysteine, reduced glutathione (GSH), glutathione peroxidase (GSH-Px), catalase (CAT), and superoxide dismutase (SOD) [2]. However, in different animal species, the indigenous antioxidant systems were insufficient to protect the spermatozoa from oxidative stress [3, 4]. Therefore, extra antioxidants supplementation was highly recommended to protect spermatozoa during liquid storage. In this respect, the positive effects of adding antioxidants at different temperature degrees to the liquid equine, sheep, and goat semen have been demonstrated in several studies [5, 6, 7].

Glutathione (L-γ-glutamyl-L-cysteinyl glycine; GSH) is a tri-peptide ubiquitously distributed in cells and it plays an important role as an intracellular defense mechanism against oxidative stress. Moreover, GSH is a principally important natural antioxidant in semen, can easily mobilize the system for the removal of peroxides by a reaction that results in the generation of oxidized glutathione [8]. In the sperm membrane, oxidized glutathione (GSSG) can reduce the mobility of sulfhydryl-containing proteins. As Sulfhydryl groups are under redox control, the change in the redox status of the membrane could be linked to ROS production that occurs during cooling and freezing-thawing of spermatozoa [9].

Taurine is one of the major non-enzymatic antioxidants. This sulfonated amino acid was found in both seminal plasma and oviductal fluid [10, 11]. When applied at an appropriate dose, it improves spermatozoa motility and displays antioxidative properties, elevating the CAT level, in association with SOD concentration [12].

Abbreviations-Cont'd

MOT %: percent motility
PMOT %: percent of sperm showing progressive motility
ROS: reactive oxygen species
TAL: tyrode albumin lactate
TEY: tris-egg yolk
SOD: superoxide dismutase
VCL: curvilinear velocity
VAP: average path velocity
VSL: straight line velocity

Cysteine is another amino acid, which has been shown to penetrate the cell membrane easily, enhancing intracellular glutathione biosynthesis both *in vitro* and *in vivo* [13]. This antioxidant has been reported to prevent loss of sperm viability, motility, and membrane integrity during liquid storage and also in the frozen state [14]. Anyhow, cysteine and taurine, as antioxidants, have been used in the cryopreservation process of humans [15], boar [16], bull [17, 18], ram [19], and goat sperm [20] to enhance the post-thaw motility and fertility of spermatozoa.

Despite the existence and the importance of all the previously mentioned studies, no reports have addressed the applications of antioxidants on motility characteristics of different goat spermatozoa types especially in the cases of thawed-frozen, fresh, and chilled sperm samples collected during breeding and non-breeding seasons. These three types are highly important and essential for the different assisted reproductive technologies. For that, the main objective of this study was to evaluate the effects of oxidized glutathione, L-cysteine, and taurine supplementation on the motility of different goat spermatozoa types assessed by computer sperm analyzer system.

Results

Table 1 shows the effects of antioxidants addition to TAL thawing medium on CASA motility parameters of frozen/thawed goat spermatozoa. Except for taurine at a concentration of 10 mM, the addition of all antioxidants in this study and all the used concentrations resulted in a significant increase ($p < 0.05$) of the MOT % parameter.

Table 2 shows the effects of adding antioxidants on fresh sperm motility characteristics. Cysteine and GSSG significantly ($p < 0.05$) increased the values of the MOT %, VAP, VSL, and VCL. When 10 mM of taurine was added, the values of VAP, VSL, and VCL were lower compared to the control, while 25 mM of this agent was able to significantly raise all the values of the analyzed motility parameters compared to control and those treated with 10 mM taurine.

Table 3 shows the effects of antioxidants addition on CASA motility parameters of chilled goat spermatozoa at 5 °C. Except for the positive effect on MOT % parameter when the spermatozoa were treated with GSSG and L-cysteine, as well as the positive effect of GSSG at 2 mM on VAP, VCL, and VSL parameters, the results showed that the antioxidants used in this study did not increase the values of different motility characteristics and no significant differences ($p > 0.05$) was noted between the values of control and the treated spermatozoa with antioxidants.

Figures 1 and 2 show the effects of the three antioxidants addition on the distribution of motility categories of both frozen/thawed and fresh samples. The positive effects were evident by increasing the percentages of rapid spermatozoa after GSSG and L-cysteine supplementations for the two concentrations. The per-

Table 1.
Effects of oxidized glutahione (GSSG), L-cysteine, and taurine on CASA motility parameters of thawed-frozen goat spermatozoa samples.

Parameter/ Treatment	MOT (%)	PMOT (%)	VAP (μm/s)	VSL (μm/s)	VCL (μm/s)
Control	57.9 + 2.5 [a]	8.4 +1.5 [a]	61.2 +2.4 [a]	38.2 + 3.1 [a]	128.1 + 6.4 [a]
GSSG 2 mM	69.9 + 1.5 [b]	10.8+0.91 [b]	69.7 + 4.4 [b]	44.7 + 2.7 [b]	140.9 + 7.9 [b]
GSSG 4 mM	64.5 + 2.9 [b]	9+1.58 [a]	63.1 +4.6 [a]	40.9 + 3.1 [a]	136.2 + 7.6 [a]
L-Cysteine 5 mM	67.8 + 3.6 [b]	12.1 +2.1 [b]	72.2 +8.1 [b]	46.2 + 2.5 [b]	145.8 + 7.6 [a]
L-Cysteine 10 mM	66 + 3.1[b]	10.3 +1.2 [b]	68.1 +5.4 [b]	43.3 + 4.3 [b]	138.1 + 6.9 [a]
Taurine 10 mM	56 +7.1 [a]	7.8 +2.1 [a]	61.6 +6.2 [a]	43.1 +4.6 [a]	134.6 + 9 [a]
Taurine 25 mM	70.3 + 3 [b]	11.7 + 1.5 [b]	72.2 +5.1 [b]	47 + 3.5 [b]	145.7 + 8.1 [b]

Mean + SD (3 replicates; for each replicate and each condition: 3 fields of 150-250 spermatozoa each were analyzed by CASA).
Data with different superscripts in the same column are significantly different ($p < 0.05$).

Table 2.
Effects of oxidized glutahione (GSSG), L-cysteine, and taurine on CASA motility parameters of fresh goat spermatozoa samples.

Parameter/ Treatment	MOT (%)	PMOT (%)	VAP (μm/s)	VSL (μm/s)	VCL (μm/s)
Control	67.9 +3.5 [a]	15.1 + 3.6 [a]	88. 7 + 8.4 [a]	61. 6 + 9.7 [a]	182.4 + 9.3 [a]
GSSG 2 mM	85.1 +5.9 [b]	25.2 + 6.2 [b]	106.9 + 11.5[b]	80.3 +17.7 [b]	205.2 +13.1 [b]
GSSG 4 mM	82.4 + 4.7 [b]	21.4 + 2.6 [b]	108 + 7.5 [b]	73.6 + 7.6 [b]	204.7 + 7.8 [b]
L-Cysteine 5 mM	84.6 +10.8 [b]	23.9 + 6.4 [b]	103.1 +16.1 [b]	73 +12.3 [b]	200 +9 [a]
L-Cysteine 10 mM	83.2 + 3.3 [b]	21.6 + 2.7 [b]	105. 9+11.6 [b]	74.1 + 12.3 [b]	195.8 +9.5 [b]
Taurine 10 mM	69.8 + 1 [a]	14.6 + 1.7 [a]	78.7 +7.1 [a]	53 + 4.7 [a]	169.7 + 8.6 [a]
Taurine 25 mM	75.3 + 4.2 [b]	18.8 + 1.6 [b]	97.2 + 8 [c]	70 + 5.8 [c]	196.4 + 8.5 [c]

Mean + SD (3 replicates; for each replicate and each condition: 3 fields of 150-250 spermatozoa each were analyzed by CASA).
Data with different superscripts in the same column are significantly different ($p < 0.05$).

Table 3.
Effects of oxidized glutahione (GSSG), L-cysteine and taurine on CASA motility parameters of chilled goat spermatozoa samples.

Parameter/ Treatment	MOT (%)	PMOT (%)	VAP (μm/s)	VSL (μm/s)	VCL (μm/s)
Control	70.2 + 1 [a]	12.4 + 1.3 [a]	71 + 3.8 [a]	46.1 + 1.9 [a]	145.2 + 7.1 [a]
GSSG 2 mM	76.3 + 3.4 [b]	13.1 + 1.5 [a]	75.3 + 2.1 [b]	49.4 +1.7 [b]	154.7 + 7.3 [b]
GSSG 4 mM	77. 6 + 4.5 [b]	11.8 + 3.3 [a]	70.8 + 3.3 [a]	45.2 + 3.7 [a]	147.7 + 8.5 [ab]
L-Cysteine 5 mM	76.6 + 4.1 [b]	12.8 + 3.2 [a]	70.5 + 4.5 [a]	44.8 + 4.2 [a]	148.4 + 6.7 [a]
L-Cysteine 10 mM	78 + 3.2 [b]	13 + 3.2 [a]	74.4 + 6 [a]	46.2 + 3.7 [a]	153.3 + 10.6 [a]
Taurine 10 mM	72 + 5.3 [a]	11.4 +1.4 [a]	68. 9 + 6.6 [a]	45.2 + 4.9 [a]	146.4 + 9 [a]
Taurine 25 mM	68.8 + 5.3 [a]	9.5 + 0.9 [a]	68.8 + 4.2 [a]	42.1 + 2.4 [a]	144.4 + 10.2 [a]

Mean + SD (3 replicates; for each replicate and each condition: 3 fields of 150-250 spermatozoa each were analyzed by CASA).
Data with different superscripts in the same column are significantly different ($p < 0.05$).

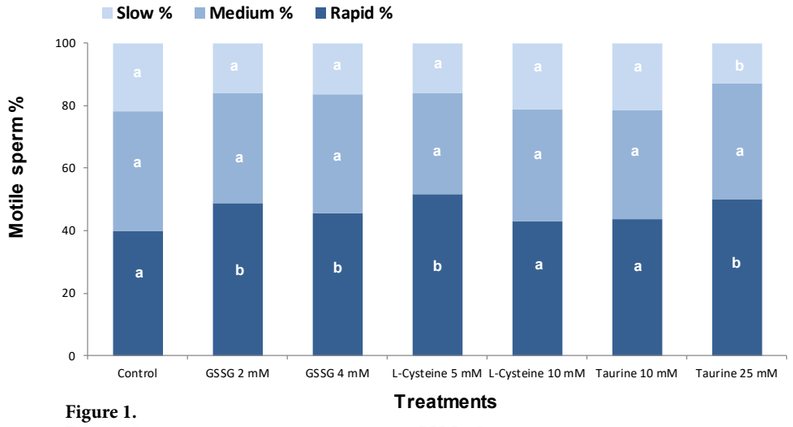

Figure 1.
Effects of antioxidants; oxidized glutahione (GSSG), L-cysteine, and taurine on the distribution of motility subpopulation of frozen/thawed goat spermatozoa samples. Different letters within each subpopulation category and between treatments significantly differ ($p < 0.05$).

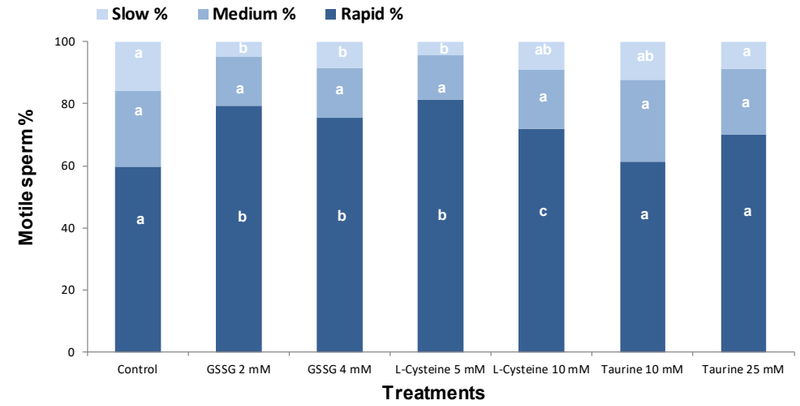

Figure 2.
Effects of antioxidants; oxidized glutahione (GSSG), L-cysteine, and taurine on the motility subpopulations of fresh goat spermatozoa samples. Different letters within each subpopulation category and between treatments significantly differ ($p < 0.05$).

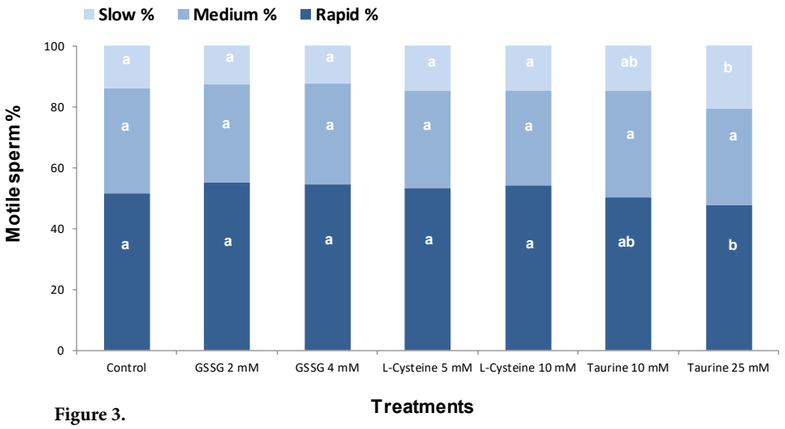

Figure 3.
Effects of antioxidants; oxidized glutahione (GSSG), L-cysteine, and taurine on the distribution of motility subpopulations of chilled goat spermatozoa samples. Different letters within each subpopulation category and between treatments significantly differ ($p < 0.05$).

centage of medium category did not differ ($p > 0.05$) between control samples and antioxidant treatments for all used concentrations. Figure 3 shows the effects of antioxidants addition on the distribution of motility categories of chilled spermatozoa samples. There were no significant differences between the control and the spermatozoa treated with GSSG and cysteine for rapid, medium, and slow categories ($p > 0.05$). When the samples were treated with taurine at a concentration of 25 mM, the percentage of rapid sperm decreased and the percentage of slow motility increased significantly ($p < 0.05$) compared to control.

Discussion

The effects of adding antioxidants on motility characteristics of three different goat spermatozoa types were investigated in the present research. The influences of the same antioxidants on frozen spermatozoa samples from different animal species were previously studied [21, 22, 23]. However, in this study, we focused on other types including frozen/thawed and unfrozen samples. The process of thawing induces ROS production in semen and if optimal supplementation of antioxidants could scavenge ROS, thus it consequently may decrease the deleterious effects of the cryopreservation process. Pieces of information on the use of antioxidants in thawing medium to improve post-thaw motility of frozen goat spermatozoa are lacking. Moreover, treating fresh and chilled semen with antioxidants during the non-breeding season may present an interesting technique in goat semen manipulations.

In this study and for the three spermatozoa types, oxidized glutathione was able to significantly increase the values of MOT % whatever the concentration was. The addition of glutathione (reduced, or oxidized) to the freezing medium of bull semen had reduced the loss of spermatozoa motility by 35 % [24]. In agreement with our results, 5 mM of GSSG had the best protective effect on ram sperm motility after semen thawing [21]. Furthermore, according to the previous authors, a toxic effect on post-thawing spermatological indicators was noted when higher concentrations (10 or 20 mM) of GSSG were used. Anyhow, it must be stressed out that GSSG may not be an efficient agent when added outside the cells, and it cannot prevent peroxidation as vitamin E [25]. The effects of GSSG in semen medium and the mechanism of action of GSSG on spermatozoa motility need more profound researches.

Our results also showed that L-cysteine had positive effects on motility parameters with relatively better results regarding the 5 mM level. Cysteine is rapidly oxidized into cystine outside the cell. Cystine and cysteine are transported into the cell by different transporters. However, cysteine had exhibited significant cryoprotective activity on post-thaw motility parameters of frozen bull spermatozoa [22]. This amino acid can improve intracellular glutathione biosynthesis and such a process may protect the membrane lipids through indirect radical scavenging properties [26]. Moreover, it was suggested that cysteine plays an antioxidative role in preventing malondialdehyde (MDA) production, resulting in higher SOD activity [22]. In contrast to our study and all the previous reports, Yildiz et al. [23] found that cysteine did not have beneficial effects on motility rates after ram semen freezing. Such variability in literature data and our study could be attributed to extender components, antioxidant dose differences, the time of spermatozoa exposure to antioxidants, and also to semen preservation protocols.

Compared to the other two antioxidants, taurine only enhanced the spermatozoa motility characteristics of fresh and frozen/thawed samples with better values for the 25 mM level. When it was applied at the same dose, this antioxidant improved ram sperm motility during cryopreservation [12]. Based on the data of Sariozkan et al. [22], taurine had a beneficial effect by raising the CAT level in the presence of ROS. In the present study, the addition of taurine to chilled spermatozoa did not cause any improvement in velocity parameters and this was similar to results obtained by Sariozkan and co-workers [22] on the bull samples. It was also reported that motility of bovine spermatozoa cooled to 5 °C was maintained close to pre-storage levels in taurine-containing citrate extender up to 48 h.

The chilled spermatozoa were less affected by antioxidants treatments than the other two spermatozoa types. In contrast, progressive sperm motility was significantly higher for chilled buck sperm samples treated by vitamin E and glutathione compared to control [27]. It should be noted that the time of incubation for the chilled samples in this study was relatively short compared to the previous study. Anyhow, a longer period of incubation at 5 - 4 °C may be necessary to demonstrate any eventual positive effects of adding these antioxidants to goat spermatozoa.

The most interesting finding from our study was the ability of antioxidants to improve motility levels of fresh goat spermatozoa collected during the non-breeding season. This type of spermatozoa may be usefully utilized in different reproductive assisted technologies after antioxidants supplementations during this critical period of reproduction. Based upon our preliminary experiments (data not shown) high levels of motility were noted from the control samples of both fresh and chilled types collected during breeding seasons and this probably was responsible for masking any significant effects of the antioxidants supplementations to such spermatozoa types during this period. Indeed, the spermatozoa show their best performances during the breeding season and normally they do not need any additional improvements concerning their motility characteristics. In contrast, the motility levels of cryo-preserved samples during the non-breeding season were very low with no clear effects of antioxidants (data not shown). These

preliminary results may have practical application in choosing when it will be the best time to add the antioxidants systems to each type of goat semen.

Our CASA system was able to distinguish three clear motile subpopulations in the different sperm samples depending on the values of the VAP parameter. Researchers have identified the existence of sperm subpopulations in species as boars, stallions, dogs, bulls, and gazelles, which were defined by specific movement characteristics [28, 29, 30]. Moreover, sperm subpopulations had different sensitivities to respond to external stimuli such as sugars, caffeine, and bicarbonate [31, 32]. Our results showed that antioxidants could not only affect the principal CASA motility characteristics, but also the distributions of spermatozoa subpopulations. The rapid spermatozoa represent the most progressive spermatozoa and it is probably suitable for being a part of the fertilizing population. This category was the most affected by antioxidants treatment especially in the cases of frozen/thawed and fresh types. In contrast, the medium subpopulation category was not influenced by the three different antioxidant treatments. It seems that this class of spermatozoa which presents a transitional stage between the rapid and slow categories may do not have sufficient time to be affected by the different antioxidants treatments. Anyhow, despite the simplicity of motility distribution of the subpopulations provides by this CASA system, the results clearly showed the importance of analyzing such distribution and which subpopulations are the most affected by antioxidants supplementations.

In the present study, we analyzed the effects of each of the three antioxidants separately; future investigations could clarify the effects of simultaneous inclusion of antioxidants' complex components on motility characteristics. In this regard, the supplementation of goat semen extenders with combinations of antioxidants had improved sperm viability and reduced oxidative stress parameters [33]. Despite the great importance of motility assessment, the beneficial effects of antioxidants on spermatozoa may not be only detectable by the evaluation of sperm motility in vitro. Further studies should include the in vitro and in vivo tests including viability, membrane integrity, and most importantly fertilization and pregnancy rates for these three spermatozoa types. Finlay, as ROS plays a very important role in the capacitation and acrosome reaction processes [34, 35]. Therefore, caution must be taken when using antioxidants to not stop these important physiological processes.

In conclusion, our data showed the advantages of adding antioxidants to thawed medium on the motility characteristics of frozen goat spermatozoa. This study also revealed that media supplementation with L-cysteine or GSSG helps in improving the motility of fresh sperm samples collected during the non-breeding season. Chilled spermatozoa collected during the non-breeding season were the less affected spermatozoa type by the different treatments. Thus, the effects of antioxidants largely depend on the temperature of sperm preservation, the used concentration, and also the type of spermatozoa pattern.

Materials & Methods

Area of study, chemicals, and media

This study was carried out at Der-Al-Hajar Animal Production Research Station, 33 km southeast of Damascus. All the chemicals were purchased from Roth (Carl Roth Gmbh-Karlsruhe-Germany). The tris-egg yolk (TEY) medium was prepared as 300 mOsm/kg solution, containing 28.5 mM g citric acid monohydrate, 29.5 mM tris (hydroxymethyl) aminomethane, and 19.8 mM glucose in 80 ml of distilled water. Twenty ml of egg yolk was then added bringing the total volume of this medium to 100 ml. The TAL solution at 300 mOsm/kg had contained 112.94 mM NaCl, 3.22 mM KCl, 0.33 mM NaH_2PO_4, 23 mM $NaHCO_3$, 0.49 mM $MgCl_2.6H_2O$, 2.04 mM $CaCl_2.2H_2O$, 1 mM $C_3H_3O_3Na$, 35 mM $C_5H_2NaO_3$ and 6 g bovine serum albumin in one liter of distilled water. The two media were held constant at pH 7.0.

Animals, semen preparation, and experimental design

Semen was obtained from five sexually-experienced Shami bucks, a native breed of Syrian goat, aged between 3 and 4 years. Semen was collected with the aid of an electro-ejaculator (Minitube - Electro Ejaculator, Germany) administrating a series of 20 cycles pulses of short electrical stimuli with each cycle (two seconds impulse, then two seconds interval) delivering a slightly higher intensity (from 0 Volt to 20 Volt maximum) until semen production. It must be noted that the same five bucks were always used in all the experiments of this study during breeding and non-breeding season. Upon collection, the semen was immediately evaluated for its general appearance and volume. For each animal and after semen collection, sperm concentration was estimated using a haematocytometer. Then an initial analysis of sperm motility was performed using the CASA system (Hamilton Thorne Biosciences, USA) whereas sperm samples at a concentration of ≥ 1 x 10^9 spermatozoa/ml were employed. All ejaculations with no or poor motility status were immediately excluded before conducting the analyses.

Three types of semen samples including fresh, chilled, and frozen were used in the present study. Semen was collected during breeding and non-breeding seasons. For both fresh and chilled samples the semen was collected during the non-breeding season in February while for the frozen samples the semen was collected during the breeding season in August. The selection of these exact seasons was based on preliminary experiments which help us on showing the most important effects of antioxidants for each type of spermatozoa in each specific season.

A total of 45 ejaculates were collected in this study. A mixture of semen from five bucks was used in each assay to diminish the effect of individual variation between the animals. It must be stressed out that the number of spermatozoa from each buck was the same in the semen mix. For the frozen spermatozoa the samples were prepared by mixing fresh semen collected during the breeding season (in August) from the five animals and diluted in a tris-based medium without egg yolk at a ratio of 1:9 (semen to tris medium, v:v) and centrifuged at 1000 × g for 15 min. The seminal plasma was discarded and the sperm pellet was suspended to a final concentration of 200 × 10^6 spermatozoa/mL with tris medium containing both filtered egg yolk (20 %, v:v) and 7 % glycerol. The diluted sperm suspensions were equilibrated at 5 °C for 3 h and loaded into 0.5 mL straws. They were then frozen in nitrogen vapor for 20 min at 10 to 15 cm of height above the nitrogen liquid and transferred to a liquid nitrogen container for storage at −196 °C until use.

Three experiments were conducted in the present study. In the first experiment, the motility characteristics of thawed spermatozoa incubated with the three antioxidants in TAL solution were analyzed. In this experiment, straws thawing were carried out by immersing the straws in a water bath at 37 °C for 30 sec. Thawed spermatozoa from 0.5 ml straws were directly incubated after thawing in 0.5 ml of TAL medium containing 0 (control), 8 and 4 mM GSSG, 10 and 20 mM L-cysteine, 20 and 50 mM taurine making the final concentration 4 and 2 mM, 5 and 10 mM, 10 and 25 mM respectively. The sperm incubation period lasted for 60 minutes at 37 °C. This experiment was replicated three times in three different weeks in August and the spermatozoa concentrations were 50 x 10^6 for the control and each treatment.

In the second experiment, sperm motility of fresh semen samples collected during the non-breeding season was analyzed. In this experiment, fresh samples of 50 x 10^6 sperm/ml were incubated in tris medium at 37 °C for 60 minutes with 0 (control), 2 and 4 mM oxidized glutathione, 5 and 10 mM L-cysteine, 10, and 25 mM taurine. This experiment was replicated three times in three different weeks in February and the spermatozoa concentrations were 50 x 10^6 for the control and each treatment.

In the third experiment, the motility of chilled spermatozoa incubated with the three antioxidants in the TEY solution was analyzed. The seminal plasma of freshly collected semen was directly removed after collection and the spermatozoa at 50 x 10^6/ml of concentration were incubated in 1 ml of TEY medium without glycerol containing 0 (control), 4 and 2 mM GSSG, 5 and 10 mM L-cysteine, 10 and 25 mM taurine for 60 minutes at 5 °C. This experiment was replicated three times in February and the spermatozoa concentrations were 50 X 10^6 for the control and each treatment.

Motility analyses

The motility characteristics were assessed using a Hamilton-Thorne motility analyzer (HTM version 12.3, Hamilton Thorne Biosciences, USA). Five microliters of diluted semen were loaded in the analyze system lame (2X-CEL of dual-sided sperm analysis chamber of 20 µm depth for the Hamilton Thorne Biosciences System), and for each sample, three fields were counted (each field counted about 150-200 spermatozoa). The motility characteristics included in the analysis were: the percent motility (MOT%), curvilinear velocity (VCL, µm/s), average path velocity (VAP, µm/s), straight-line velocity (VSL, µm/s), and the percent of sperm showing progressive motility (PMOT %: VAP ≥ 50 µm/s and STR ≥ 80 %).

Moreover, spermatozoa subpopulations were defined by the Hamilton Thorne CASA system in three categories, each of the categories is assigned an arbitrary number from 3-1 increasing correspondingly from the lowest to the highest velocity; Rapid (3): fraction of all cells moving with VAP > path velocity (VAP = 25 µm/s), Medium (2): fraction of all cells moving with VAP cutoff (5 µm/s) < VAP < path velocity (VAP = 25 µm/s), Slow (1): fraction of all cells moving with VAP < VAP cutoff (5 µm/s) or VSL < VSL cutoff (11µm/s).

The HTM settings used for goat spermatozoa were negative phase contrast optics at a recording rate of 60 frame/s, light adjustment 50-110, minimum contrast 70, minimum cell size 5 pixels, non motile head size 10 pixels, non motile head intensity 80, low VAP cut off 20mm/s, low VSL cut off 5 mm/s, static size limit 0.60/4.32 (min/max), static intensity limit 0.20/1.92 (min/max), static elongation 7/91 (min/max).

Statistical analysis

Minitab program (Minitab Coventry, United Kingdom) was used for the statistical analysis. The normality of values distribution was first tested with the Shapiro-Wilk test. Data regarding the different antioxidants effects on motility values were subjected to a factorial analysis of variance (ANOVA, general linear model procedure, GLM) followed by multiple pairwise comparisons using a post-hoc (*Tukey's* test). The results are presented as mean + SD and the threshold of signification was set at $p < 0.05$.

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Competing Interests

The author declares that there is no conflict of interest.

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